CMI CLINICAL MICROBIOLOGY AND INFECTION
ESCMID

Mechanisms of resistance in clinical isolates of Pseudomonas aeruginosa less susceptible to cefepime than to ceftazidime.

Journal:	*Clinical Microbiology and Infection*
Manuscript ID:	CLM-10-2802.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	CAMPO ESQUISABEL, ANA BELEN; HOSPITAL SIERRALLANA, SERVICIO DE MICROBIOLOGIA; Univ Hosp Marques Valdecilla, Microbiology Rodríguez, María Cruz; Instituto de Biomedicina y Biotecnología de Cantabria, IBBTEC Alain, Ocampo-Sosa; Univ Hosp Marques Valdecilla, Microbiology Rodríguez, Cristina; Univ Hosp Marques Valdecilla, Microbiology Martinez-Martinez, Luis; Univ Hosp Marques Valdecilla, Microbiology
Key Words:	Pseudomonas aeruginosa , cefepime, resistance, efflux pumps, PSE-1
Abstract:	The MIC of cefepime determined with the MicroScan WalkAway system was >=2 times higher than that of ceftazidime for 105 clinical isolates of Pseudomonas aeruginosa. This phenotype was confirmed by reference microdilution in 68 (64.8%) isolates, corresponding to 48 different rep-PCR patterns. The PSE-1 b-lactamase was identified in only 13.2% isolates, while oxacillinases were not identified in any of the 68 isolates. The level of expression of mexB, mexD and mexY was determined by real time RT-PCR in 8 clinical isolates representative of the different clones and patterns of susceptibility to cefepime and ceftazidime and in strain PAO1. All clinical strains overexpressed the mexY gene (18.3 to 152.7-fold in comparison with PAO1), although there was not a linear relationship between MIC of cefepime and level of mexY expression. Five of these strains contained mutations in the regulatory gene mexZ. mexD and mexB were also overexpressed in 3 and 2 isolates, respectively. Different mutations were observed in the regulatory genes nalD, mexR, nfxB and nalC. In conclusion, we have documented in our institution a polyclonal spread of P. aeruginosa with higher MICs of cefepime than to ceftazidime, related to overexpression of MexXY-OprM, coincident in some isolates with the production of PSE-1, MexCD-OprJ or MexAB-OprM.

INTRODUCTION

MICs of cefepime against wild-type clinical isolates of *Pseudomonas aeruginosa,* are usually similar to those of ceftazidime [1]. However, strains intermediate or resistant to cefepime and susceptible to ceftazidime have been described [2-7]. This phenotype has been related to hyperproduction of the efflux system MexXY-OprM [2, 5-7] and/or the production of OXA-31 [3], OXA-35 [4] or PSE-1 [2, 7]. Although the MexCD-OprJ system does not contribute to the basal natural resistance of *P. aeruginosa* [8], when it is overexpressed it causes increased resistance to cefepime and other compounds (fluoroquinolones, macrolides,...) [9]. However, MexCD-OprJ overexpression is uncommon among clinical isolates of *P. aeruginosa*, with the notable exception of isolates from cystic fibrosis patients. In a previous study, none of 38 isolates more resistant to cefepime than to cefazidime overexpressed the MexCD-OprJ [2]. Also, in a collection of bacteremic isolates, MexAB-OprM and MexXY-OprM were overexpressed in 11% and 36% of the isolates, respectively, while MexCD-OprJ overexpression was not detected in any isolate [10]. Similarly, only 4 out of 110 clinical isolates with decreased susceptibility to ciprofloxacin hyperproduced MexCD-OprJ, and although these 4 isolates were less susceptible to cefepime than to ceftazidime, it was not possible to demonstrate the direct involvement of this efflux system in the decreased activity of cefepime [11].

On the other hand, susceptibility testing of *P. aeruginosa* to cefepime with several automatic systems has caused either major errors (false resistance) or minor errors (resulting from categorizing some isolates as intermediate to cefepime when they actually were susceptible to this compound) [12-17].

In our center, using the MicroScan WalkAway system (Dade Behring, Sacramento, CA), we have obtained during the last years clinical isolates of *P. aeruginosa* presenting lower susceptibility to cefepime than to ceftazidime. The objectives of this study were to confirm this phenotype using a standardized susceptibility testing assay and to describe the molecular mechanisms involved.

MATERIAL AND METHODS.

Bacteria. One hundred and five consecutive clinical isolates of *P. aeruginosa* isolated in the period September 2004 to June 2005 for which the MIC of cefepime was >=2 times higher than that of ceftazidime were collected from 99 patients (64 males and 35 females). During the study period, 1652 isolates of *P. aeruginosa*, cultured from 780 patients were obtained in our centre; the 105 isolates we initially selected represent a 6.4% of all isolates, and the 99 patients correspond to 12.7% of all patients from whom *P. aeruginosa* was isolated. The organisms were cultured from wound exudates (n=44), respiratory samples (n=25), urines (n= 21), blood cultures (n=3) or others (n=12, including lung biopsy, semen and sterile fluids).

Identification and susceptibility testing of the bacteria were performed with the MicroScan Walkaway 96 system, using Combo Neg 1S panels. Isolates were maintained at –80ºC in tryptic soy broth with 10% glycerol. Organisms were plated out twice on Mueller-Hinton agar plates (Difco) before additional studies were performed.

Susceptibility testing. MICs of both cefepime (Bristol Myers Squibb, Madrid, España) and ceftazidime (Sigma, Madrid, España) against the indicated 105 isolates were determined by microdilution, according to CLSI guidelines [18]. *P. aeruginosa* ATCC 27853 was included as a control. The microdilution assay was performed three times on three different days. The final MIC values considered in this study were those corresponding to the modal values for every isolate. Cefepime and ceftazidime susceptibilities of the 105 isolates were also determined by the disk-diffusion assay (CLSI guidelines), using 30 µg disks (BD BBL Sensi-DiscTM, USA).

Molecular typing. Clonal relationship of the isolates was evaluated by REP-PCR, using primers described by Vila et al. [19]. Amplicons were analysed by agarose gel electrophoresis. Two isolates were considered clonally unrelated when two or more different bands were observed. Additionally, pulsed-field gel electrophoresis (PFGE) was performed in organisms presenting the same banding pattern or with only 1 band of difference. DNA agarose plugs were digested overnight with *Spe*I (Roche Diagnostics, Indianapolis, IN, USA) at 37℃. The restriction fragments were separated on 1% w/v agarose gels in 0.5% TBE buffer in a CHEF-DR II electrophoresis system (Bio-Rad Laboratories, Richmond, CA, USA) for 26 h at 14ºC using a pulse ramping rate changing from 1 to 20 s at 6 V/cm. PFGE patterns were interpreted according to the criteria by Tenover et al. [20].

Analysis of resistance mechanisms. Detection of genes coding for oxacillinases of groups I, II y III, and penicillinase PSE-1 was performed by PCR in the 68 isolates for which the phenotype herein investigated was confirmed by reference microdilution (see below), using previously reported primers and

conditions [21, 22]. Amplicons (both strands) were sequenced using an external resource (Macrogen, Korea).

The level of expression of *mexB, mexD* and *mexY* was determined by real time PCR (RT-PCR) in strain PAO1 and in 8 clinical isolates (representative of the different clones and patterns of susceptibility to cefepime and ceftazidime), including 5, 2 and 1 isolates for which the MIC of cefepime was higher than that of ceftazidime 2, 4 or 16 times, respectively (Table 1). Total RNA was extracted with the Rneasy Protect bacteria Mini Kit (QIAGEN, Hilden, Alemania), according to manufacturer's recommendations. DNA was removed with RNase-Free DNase treatment and RNA preparations were tested for the lack of *Pseudomonas aeruginosa* DNA contamination by PCR with primers specific for the *rpsL Pseudomonas* gene. None of the RNA samples used in the study amplified before cycle 35. ARN concentration was determined spectrophotometrically and the quality of the samples was checked in agarose gels in denatured conditions. Quantification of specific genes was performed by qPCR in a two-step reverse transcription and real-time PCR using the iScript cDNA synthesis kit and iQ SYBR Green supermix (Bio-Rad). The *rpsL* gene was used as an internal reference to normalize the relative amount of RNA. The experiment was repeated in triplicate, using independent RNA extractions in every assay. The expression of the indicated genes was compared to their expression in the PAO1 strain. The genes were considered to be overexpressed when the amount of RNA was at least 4 times higher than that in PAO1.

In the same set of 8 strains, the sequences of the regulatory genes *mexR*, *mexZ*, *nfxB*, *nalC* and *nalD* were determined after their amplification by PCR,

using the primers indicated in table 2. Amplicons were sequenced, and the BLAST program was used to compare the nucleotide and protein sequences to those available on the www.ncbi.nlm.nih.gov website.

RESULTS AND DISCUSSION.

Reference microdilution confirmed that the MICs of cefepime were ≥3, 2 and 1 twofold dilutions higher than that of ceftazidime for 10 (9.5%), 35 (33.3%) and 23 (22%) of the corresponding isolates. However, in 37 (35.2%) isolates the MICs of cefepime were the same or 1 dilution lower that those of ceftazidime. Interestingly, differences in MIC of cefepime by the reference and the commercial method did not translate into either very major (false susceptibility) or major (false resistance) errors, but in 5 (4.8%) isolates caused a minor error (intermediate by reference microdilution and resistance by the automatic system). The same clinical categories were obtained with microdilution and with the standardized disk diffusion method, except for one isolate susceptible to cefepime by microdilution and resistant by disk-diffusion and two other isolates susceptible to cefepime by microdilution and intermediate by disk-diffusion. These results support that identification of *P. aeruginosa* strains more resistant to cefepime than to ceftazidime with the MicroScan WalkAway system is not completely reliable. This agrees with multiple reports [12-17] indicating the difficulties of automatic systems when testing cefepime against *P. aeruginosa*.

There is scarce information on the clinical aspects related to *P. aeruginosa* less susceptible to cefepime than to ceftazidime. Our isolates were obtained from a variety of clinical samples, as already documented in another study [7]. The use of piperacillin-tazobactam has been reported as an independent risk factor for the acquisition of *P. aeruginosa* with this phenotype [7]. It is possible that the use of quinolones, which are good substrates for different efflux pumps [8], may also select for strains with this phenotype.

The combined typing strategy of Rep-PCR/PFGE allowed the identification of 47 different clones, and organisms of the same clone presented similar (±1 twofold dilution) MICs of cefepime and ceftazidime. This polyclonal situation contrast with the results from another study in Spain, documenting the expansion of a single clone affecting 51 patients [7].

Oxacillinase genes were not detected, while the blaPSE-1 gene was amplified in 9 (13.2%) isolates. This indicates that the polyclonal nature of our isolates is not due to horizontal transmission of the genes encoding PSE-1 or an oxacillinase. The possible contribution of AmpC hyperexpression in our isolates was ruled out testing (by disk-diffusion) both ceftazidime and cefepime in media with or without cloxacillin (Sigma, 250 mg/l). The inhibition zones of cefazidime did not change (>=5 mm) in the presence of cloxacillin for any of the strains, while a change was observed for cefepime in only three strains (none of which were any of the 8 isolates studied in detail).

The 8 isolates studied in detail, overexpressed *mexY*. However, there was not a clear relationship between the level of *mexY* expression and the MIC of cefepime. Only 3 strains showed significant levels of expression of *mexD* with MIC values of ceftazidime/cefepime ranging from 2-8/4-16 µg/ml (Table 1); These strains lack PSE-1 but as they also displayed increased expression of *mexY*, it is rather difficult to define the actual contribution of either (or even both) efflux systems in the observed phenotypes. These three isolates overexpressing both *mexY* and *mexD* had different MIC values of cefepime and were not the ones with the highest MICs of cefepime suggesting that in addition of the (plausible) role of MexXY-OprM and perhaps that of MexCD-OprJ, other factors should be related in the strains we studied. *mexB* was also

overexpressed in 2 strains, but this overexpression is likely unrelated to the studied phenotype (Table 1).

Among the 8 strains studied in detail, HUMV_038 and HUMV_110 expressed PSE-1, but it is difficult to demonstrate whether their susceptibilities to ceftazidime and cefepime are due to MexXY efflux activity or PSE-1 expression. Several mutations at DNA and protein levels were observed in all the efflux systems regulators we studied. In MexR, the Val-126Glu substitution was found in 7 out of the 8 strains (Table 1). This previously described mutation [23-25] was considered non significant as it was also observed in susceptible wild-type strains. Strain HUMV_026 carried a Thr-130Pro substitution. A similar amino acid substitution at the same position (Thr-130Ser) was described before [25] with no significant differences between susceptible and resistant isolates. A frameshift mutation due to an insertion of 12 bp between nucleotides 400 and 401 was also discovered in strain HUMV_026. This mutation affects the C-terminal portion of the protein including the stop codon TAA found in PAO1, resulting in a larger peptide. Frameshit mutations in MexR have already been reported related to MexAB-OprM overexpression [23, 26, 27]. Nevertheless, this change does not seem to considerably affect the ability of MexR to bind the promoter region of *mexAB-oprM* nor the way the repressor protein modulates the expression of this operon, as no high levels of expression were registered for *mexB* in strain HUMV_026.

NalC, the repressor of ArmR (antirepressor for MexR) displayed 3 recurrent amino acid substitutions: Gly-71Glu, Glu-153Gln and Ser-209Arg. Such mutations have also been described [26] and they seemed not to affect the integrity of NalC. Three new amino acid substitutions were observed in NalC:

Ala-145Val, Pro-210Lys, Ala-186Thr, but we could not associate them with changes in expression of *mexB*. In strain HUMV_038, *nalC* shows two nucleotide deletions at positions 123 and 147 generating a frameshift affecting the N-terminal portion of the protein, which contains the DNA binding domain (from residues 20-64). The new protein has 149 amino acids residues instead of the 213 of the PAO1 strain. A 1-bp insertion in the *nalC* gene of strain HUMV_026 at position 611 generates a frameshift mutation that affects the C-terminal extreme of the protein from residue Lys-203. Strain HUMV_039 has 1 nucleotide deletion in *nalC* at position 586 creating a frameshift that affects its C-terminal portion of the protein from residue Ser195. It is difficult to correlate the effects of the single base changes in *nalC* of HUMV_026 and HUMV_039 with changes in expression of MexB. In strain HUMV_110 the combination of mutation in both proteins (MexR: Val-126Glu; NalC: Gly-71Glu, Glu-153Gln and Ser-209Arg) may be related to the increased levels of MexB expression.

Mutations in *nalD* were only observed in strain HUMV_038, which displayed a 16 bp deletion at nucleotide 85 and a 3 bp deletion at position 101. Both deletions affect the N-terminal portion of the protein that contains the DNA binding domain, generating a truncated peptide. These changes together with those found in NalC may be responsible for MexB overexpression in this strain. A previous study also showed that combined action of mutations in *mexR*, *nalC* and *nalD* may be responsible for the overexpression of MexAB-OprM [25].

mexZ presented several mutations in 5 out of 8 strains. Several attempts to amplify *mexZ* in strain HUMV_026 were unsuccessful, suggesting that this strain lacks this gene, possibly due to a deletion. This can also explain the overexpression of MexY. Two isolates (HUMV_038 and HUMV_110) had a

substitution of 1 nucleotide at position 60 (GAG for TAG), which generates a premature stop codon resulting in a truncated peptide, lacking the first 28 residues where a fragment of the DNA binding domain (from residues 15-60) is present in wild-type strains. This mutation could correlate with MexY overexpression (Table 1). The *mexZ* gene of strain HUMV_039 has an insertion of 71 bp between nucleotides 321 and 322. This sequence is almost identical (only differs in 1 bp) to a sequence placed immediately upstream, flanked by two imperfect direct repeats of 12 bp G(C)GGT(C)GCTGGACA that most likely caused such sequence duplication. This mutation could also explain the high levels of expression of *mexY* (93.3-fold higher) when compared to that of PAO1 (Table 1). The strain HUMV_072 presented an insertion of 1 bp in *mexZ* between nucleotides at positions 591 and 592. This insertion leads to a frameshift that affects the C-terminal extreme of MexZ from Asp-297 and may explain the high levels of expression of *mexY* and consequently the MIC values of cefepime (16 µg/ml) observed in this strain (Table 1). The Gly-195Arg substitution is similar to a formerly described (Gly-195Glu) that correlated with overexpression of *mexY* and high MIC values of FEP in clinical strains of *P. aeruginosa* [2]. Other mutations observed in MexZ were single amino acid substitutions: Leu-162Arg (strain HUMV_039), and Gly-137Asp plus Gly-195Arg (strain HUMV_111). The *mexZ* gene of strains HUMV_089 and HUMV_057 did not show any mutations, however *mexY* was overexpressed in both strains (Table 1), which may be due to mutations in additional mediators that control the expression of *mexXY*, as previously suggested in other studies [26, 28, 29]. Several different amino acid substitutions in NfxB, the repressor of MexCD-OprJ, have been identified. The amino acid substitutions Arg-33His and Asp-

68Gly were previously described in clinical strains of *P. aeruginosa* [25, 30] but with different residue numbers (Arg-21His and Asp-56Gly, respectively). This is probably due to a miss-annotation of *nfxB*, hence the differences in the residues positions reported. In this study we have assumed that the start codon of the *nfxB* gene might be 36 nts upstream the "proposed" ATG, considering that the sequence AGGCCAG within the bounds of the gene (12 bp upstream the ATG) is more likely to be a ribosome binding site of this gene. In addition, the size of this "corrected sequence" is similar to that of its homolog genes in other *P. aeruginosa* strains like PA7 or even to that of other pseudomonads like *P. syringae* B728a. These two amino acid substitutions (Arg-21His and Asp-56Gly) were found to be related to ciprofloxacin resistance [30]. In the strain HUMV_057 a deletion of 1 bp in *nfxB* at position 541 leads to a frameshift mutation affecting the C-terminal portion of NfxB from residue N180. Beside this there is a single base pair substitution at position 600, affecting the stop codon (TGA for TGG), which translates in a protein larger than the one in the wild type PAO1. Interestingly, mutations in the stop codon, leading to a longer predicted NfxB, have also been documented among MexCD-OprJ hyperproducing mutants in vitro and in animal models of infection [31].

ACKNOWLEDGMENTS.

This study was supported by Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008).

CONFLICT OF INTERESTS.

None to declare.

REFERENCES.

1. Vila J, Marco F. [Interpretive reading of the non-fermenting gram-negative bacilli antibiogram.]. Enferm Infecc Microbiol Clin 2010; 23. [Epub ahead of print].

2. Hocquet D, Nordmann P, El Garch F, et al. Involvement of the MexXY-OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2006; 50 (4): 1347–51.

3. Aubert D, Poire L, Chevalier J, et al. Oxacillinase-mediated resistance to cefepime and susceptibility to ceftazidime in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2001; 45 (6): 1615–20.

4. Aubert D, Poirel L, Ali AB, Goldstein FW, Nordmann P. OXA-35 is an OXA-10-related beta-lactamase from *Pseudomonas aeruginosa*. J Antimicrob Chemother 2001; 48(5): 717-21.

5. Somvadee L. Expression of the MexXY-OprM efflux system in *Pseudomonas aeruginosa* with discordant cefepime/ceftazidime susceptibility profiles. Infection and drug resistance 2008; 1: 51-55.

6. Baum EZ, Crespo-Carbone SM, Morrow BJ, Davies TA, Foleno BD, He W, Queenan AM, Bush K. Effect of MexXY overexpression on ceftobiprole

susceptibility in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2009; 53 (7): 2785-90.

7. C. Peña, C. Suarez, F. Tubau, C. Juan, B. Moya, M. A. Dominguez, A. Oliver, M. Pujol, J. Ariza. Nosocomial Outbreak of a Non-Cefepime-Susceptible Ceftazidime-Susceptible *Pseudomonas aeruginosa* Strain Overexpressing MexXY-OprM and Producing an Integron-Borne PSE-1 ß-Lactamase. Journal of Clinical Microbiolog 2009: 47 (8): 2381-2387.

8. Nikaido H, Zgurskaya HI. Antibiotic efflux mechanisms. Curr Opin Infect Dis. 1999; 12(6): 529-36.

9. Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsijimoto, and T. Nishino. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXYOprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother 2000; 44: 3322–3327.

10. Hocquet D, Berthelot P, Roussel-Delvallez M, Favre R, Jeannot K, Bajolet O,Marty N, Grattard F, Mariani-Kurkdjian P, Bingen E, Husson MO, Couetdic G,Plésiat P. *Pseudomonas aeruginosa* may accumulate drug resistance mechanisms without losing its ability to cause bloodstream infections. Antimicrob Agents Chemother 2007; 51(10): 3531-6.

11. Jeannot K, Elsen S, Köhler T, Attree I, van Delden C, Plésiat P. Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD-OprJ efflux pump. Antimicrob Agents Chemother 2008; 52(7): 2455-62.

12. Helio S. Sader, Thomas R. Fritsche, Ronald N. Jones. Accuracy of three automated systems (MicroScan WalkAway, VITEK, and VITEK 2) for susceptibility testing of *Pseudomonas aeruginosa* against five broad-spectrum beta-lactam agents. J Clin Microbiol 2006; 44 (3): 1101-1104.

13. Stefan Juretschko, Vincent J. LaBombardi, Stephen A. Lerner, Paul C. Schreckenberger, and the *Pseudomonas* AST Study Group. Accuracies of β-lactam susceptibility test results for *Pseudomonas aeruginosa* with four automated systems. J Clin Microbiol 2007; 45 (4): 1339-1342.

14. Juretschko S, Labombardi VJ, Lerner SA, Schreckenberger PC; Pseudomonas AST Study Group. Accuracies of beta-lactam susceptibility test results for *Pseudomonas aeruginosa* with four automated systems (BD Phoenix, MicroScan WalkAway, Vitek, and Vitek 2). J Clin Microbiol. 2007; 45(4): 1339-42.

15. Joyanes P, del Carmen Conejo M, Martínez-Martínez L, Perea EJ. Evaluation of the VITEK 2 system for the identification and susceptibility testing of three species of nonfermenting gram-negative rods frequently isolated from clinical samples. J Clin Microbiol 2001; 39(9): 3247-53.

16. Biedenbach DJ, Marshall SA, Jones RN. Accuracy of cefepime antimicrobial susceptibility testing results for *Pseudomonas aeruginosa* tested on the MicroScan WalkAway System. Diagn Microbiol Infect Dis 1999; 33(4): 305-7.

17. Jones RN, Biedenbach DJ, Marshall SA, Pfaller MA, Doern GV. Evaluation of the Vitek system to accurately test the susceptibility of *Pseudomonas aeruginosa* clinical isolates against cefepime. Diagn Microbiol Infect Dis. 1998; 32(2): 107-10.

18. Clinical Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Eighth Edition. M07-A8. CLSI, 2009. Wayne, Pa.

19. Vila, J., M. A. Marcos, M. T. Jiménez de Anta. A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus-A. baumannii* complex. J. Med. Microbiol; 1996. 44 (6): 482-489.

20. Tenover FC, A. R., Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restricition patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin. Microbiol 1995; 33, 2233-223.

21. Seungok Lee, Yeon-Joon Park, Myungshin Kim, Hae Kyung Lee, Kyungja Han, Chang Suk Kang, Moon Won Kang. Prevalence of Ambler class A and D

b-lactamases among clinical isolates of *Pseudomonas aeruginosa* in Korea. J. Antimicrob Chemother 2005; 56, 122–12.

22. Gutiérrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, Pérez JL, Oliver A. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. Antimicrob Agents Chemother 2007; 51(12): 4329-35.

23. Ziha-Zarifi, I., C. Llanes, T. Köhler, J.-C. Pechère, P. Plesiat. In vivo emergence of multidrug-resistance mutants of *Pseudomonas aeruginosa* overexpressing the active afflux systems MexA-MexB-OprM. Antimicrob. Agents Chemother 1999; 43: 287-291.

24. Pai, H., J. Kim, J.H.Lee, K.W. Choe, N. Gotoh. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother 2001; 45: 480-484.

25. Kiser, T.H., M.D. Obritsch, R. Jung, R. MacLaren, D. N. Fish. Efflux pump contribution to multidrug resistance in clinical isolates of *Pseudomonas aeruginosa*. Pharmacotherapy 2010; 30: 632-638.

26. Llanes, C., D. Hocquet, C. Vogne, D. Benali-Baitich, C. Neuwirth, and P. Plesiat. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob. Agents Chemother 2004; 48: 1797–1802.

27. Srikumar, R., C.J. Paul, K. Poole. Influence of mutation in the mexR repressor gene on expression of the MexAB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol 2000; 182: 1410-1414.

28. Sobel, M. L., G. A. McKay, and K. Poole. Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother 2003; 47: 3202–3207.

29. Vogne, C., J. R. Aires, C. Bailly, D. Hocquet, and P. Ple´siat. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob. Agents Chemother 2004; 48: 1676–1680.

30. Higgins, P.G., A.C, Fluit, D. Milatovic, J. Verhoef, F.-J. Schmitz. Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. Int. J. Antimcrob. Agents 2003; 21 (5): 409-13.

31. Mulet X, M. D. Maciá, A. Mena, C. Juan, J. L. Pérez, and A. Oliver. Azithromycin in *Pseudomonas aeruginosa* Biofilms: Bactericidal Activity and Selection of nfxB Mutants. Antimicrob. Agents Chemother 2009; 53 (4): 1552–1560.

Table 1. MICs of ceftazidime and cefepime against clinical strains of *Pseudomonas aeruginosa* and expression of efflux pump genes and amino acid variations in regulatory proteins of the corresponding organisms.

	MIC (µg/ml)		Efflux pumps expression			*bla* *PSE-1*	Amino acid substitutions/mutations in regulatory proteins				
Strain	CAZ	FEP	*mexB*	*mexD*	*mexY*		MexZ	NalD	MexR	NfxB	NalC
HUMV_038	2	4	5.64	1	95.3	+	nt 60T-G	nt 85Δ16 nt 101Δ3	$V_{126}E$	R_5G $K_{144}I$	$G_{71}E$ $E_{153}Q$ nt 123Δ1 nt t147Δ1
HUMV_039	2	4	1.08	18.1	93.3	-	$L_{162}R$ nt 321 71 nt322	wild-type	$V_{126}E$	$R_{33}H$ $D_{68}G$	$G_{71}E$ $A_{145}V$ nt 586Δ1
HUMV_089	4	8	0.96	2	54.9	-	wild-type	wild-type	$V_{126}E$	w.t	$G_{71}E$
HUMV_026	8	16	2.09	4	26.8	-	Not amplified	wild-type	$V_{126}E$ $T_{130}P$ nt 400 12 n t 401	$R_{33}H$ $D_{68}G$ K_{144} R $N_{195}I$	$G_{71}E$ $A_{145}V$ nt 611 1 nt 612
HUMV_110	8	16	5.41	2.4	186.8	+	nt 60T-G	wild-type	$V_{126}E$	R_5G	$G_{71}E$ $E_{153}Q$ $S_{209}R$
HUMV_057	4	16	0.81	150.5	18.3	-	wild-type	wild-type	w.t	$R_{54}C$ nt 541Δ1 nt 600A-G	$G_{71}E$ $S_{209}R$ $P_{210}L$
HUMV_111	16	64	1.1	1.7	152.7	-	$G_{137}D$ $G_{195}R$	wild-type	$V_{126}E$	w.t	$G_{71}E$
HUMV_072	1	16	1.0	2.2	140	-	nt 591 1 nt 592	wild-type	$V_{126}E$	T_7N	$G_{71}E$ $A_{186}T$

Table 2. Primers for amplification and expected amplicon sizes of genes involved in efflux systems regulation.

Efflux system	Gene	Primers	Sequence (5´-3´)	Expected size (pb)
MexCD-OprJ	*nfxB*	NfxB_F	AAATGATCTTTTGACAGCTAATTCCT	760
		NfxB_R	ACTGATCTTCCCGAGTGTCG	
MexAB-OprM	*mexR*	MexR_F	TGTTCTTAAATATCCTCAAGCGG	729
		MexR_R	GTTGCATAGCGTTGTCCTCA	
	nalC	NalC_F	TCAACCCTAACGAGAAACGCT	813
		NalC_R	TCCACCTCACCGAACTGC	
	nalD	NalD_F	GCGGCTAAAATCGGTACACT	788
		NalD_R	ACGTCCAGGTGGATCTTGG	
MexXY-OprM	*mexZ*	MexZB_F	CCCTTGTGAGGACGTTCA	968
		MexZB_R	CCCAGAGCGATTGCAGATA	